

Announcements

- Mid-term feedback is in the works at this time
- Chapter 9 post-lab write-up due week of Mar 25 – 30

Chapter 10: Overview

- **Week 1 – 10A:**
 - Isolate and wash erythrocytes and plasma membrane fractions (washing & centrifugation)
- **Week 2 – 10BC:**
 - Determine yield of membrane, lipids, and composition of lipids (TLC & cholesterol assay)
- **Week 3 – 10D:**
 - Separation of proteins by SDS-PAGE and immunoblotting
- **Week 4 – 10E:**
 - Visualization of SDS-PAGE and immunoblot results (*i.e.* Western Blot analysis)

Chapter 10A: Lipids and Membranes

Objectives

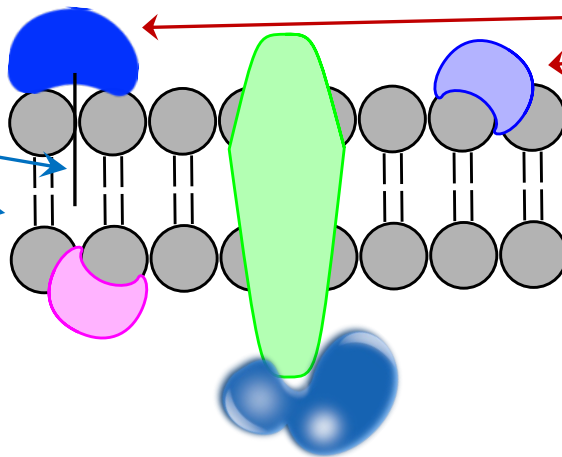
- Understand lipid membrane composition, structure, and function
- To isolate red blood cells from bovine (*Bos taurus*) blood
- Prepare washed and unwashed membrane fractions

Procedures

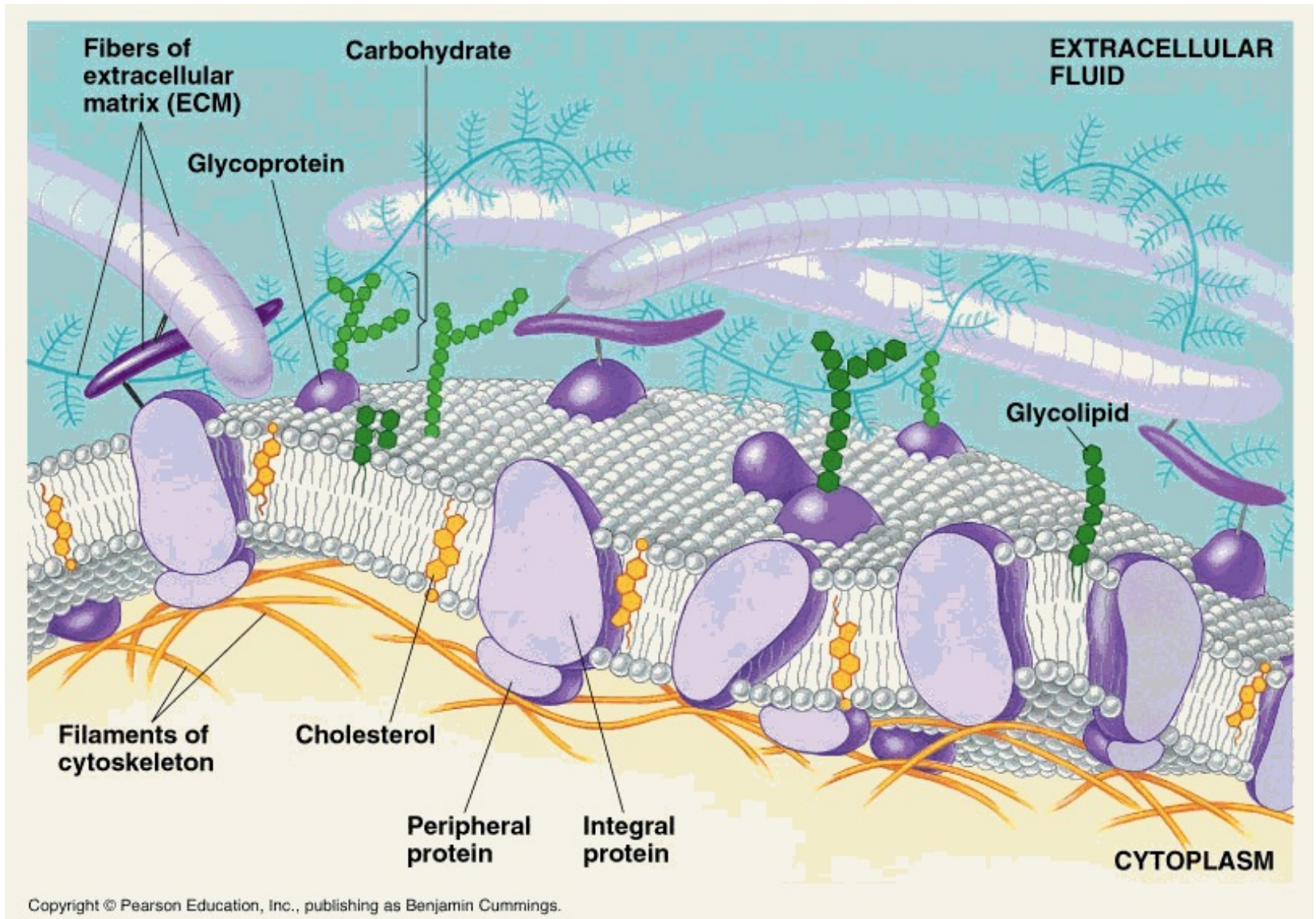
- **Isolate erythrocytes** from fresh bovine blood by **centrifugation**
- **Lyse erythrocytes** via **osmotic shock using low-salt buffer**
- To fractionate membrane preparations containing peripheral bound proteins away from integral membrane proteins
- **Lyophilize** (freeze dry) membrane preparations for next week's lipid extraction

Plasma Membrane

- Physical barrier between cell and external environment
- Separates organelles and subcellular structures
- Composed of lipids, proteins, and carbohydrates
- Exists in a *fluid-mosaic model*
 - Lipids expose **hydrophilic** head to aqueous environment
 - **Hydrophobic** lipid tails are internal to membrane
 - Lipid-bilayer embedded with proteins



Plasma Membrane

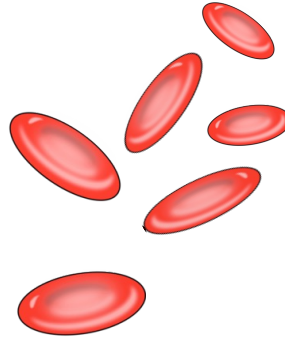


Erythrocyte Membranes

- **Red Blood Cells – Erythrocytes**

- Human Erythrocyte Membrane:

- 50% protein
- 40% lipid
- 10% carbohydrate



- Isolated as “ghosts” –remains after removing hemoglobin and intracellular components
- Isolated from whole blood extract through ***cell lysis and centrifugation***
- Do not contain other organelles – easier sample preparation

Isolation of Membranes

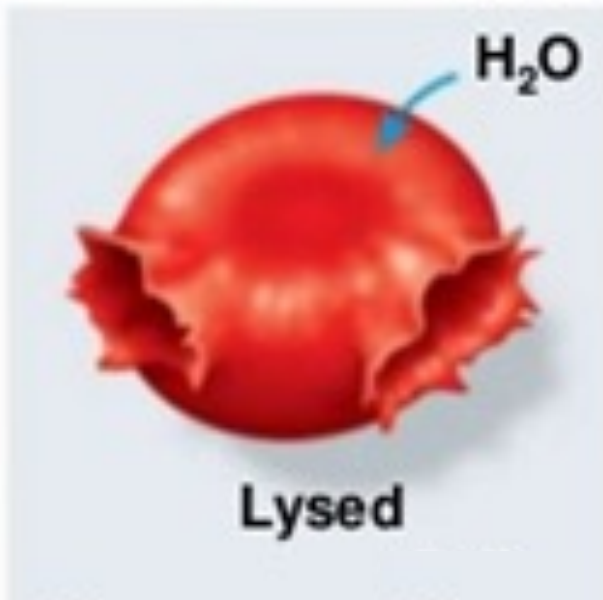
- Red Blood Cells **ISOLATED** from blood with low-speed centrifugation using Phosphate Buffered Saline (**PBS**)
 - Platelets and lymphocytes (white blood cells) float to top, RBCs to bottom
- RBCs are **LYSED** using Hypotonic Phosphate Buffered Saline (**HPBS**)
 - Low osmotic pressure in HPBS buffer – cells swell and rupture due to hypotonic solution
 - Only plasma membrane remains, because no organelles in RBCs

How does HPBS rupture erythrocytes?

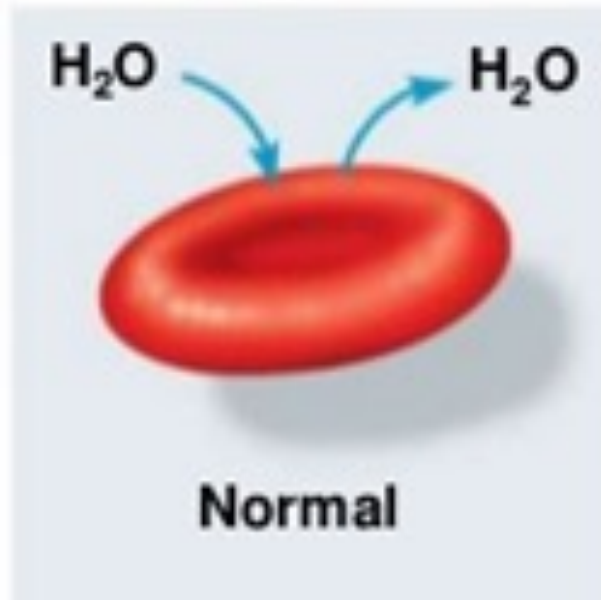
Osmolysis

If EXTRACELLULAR ENVIRONMENT is:

Hypotonic



Isotonic



Hypertonic

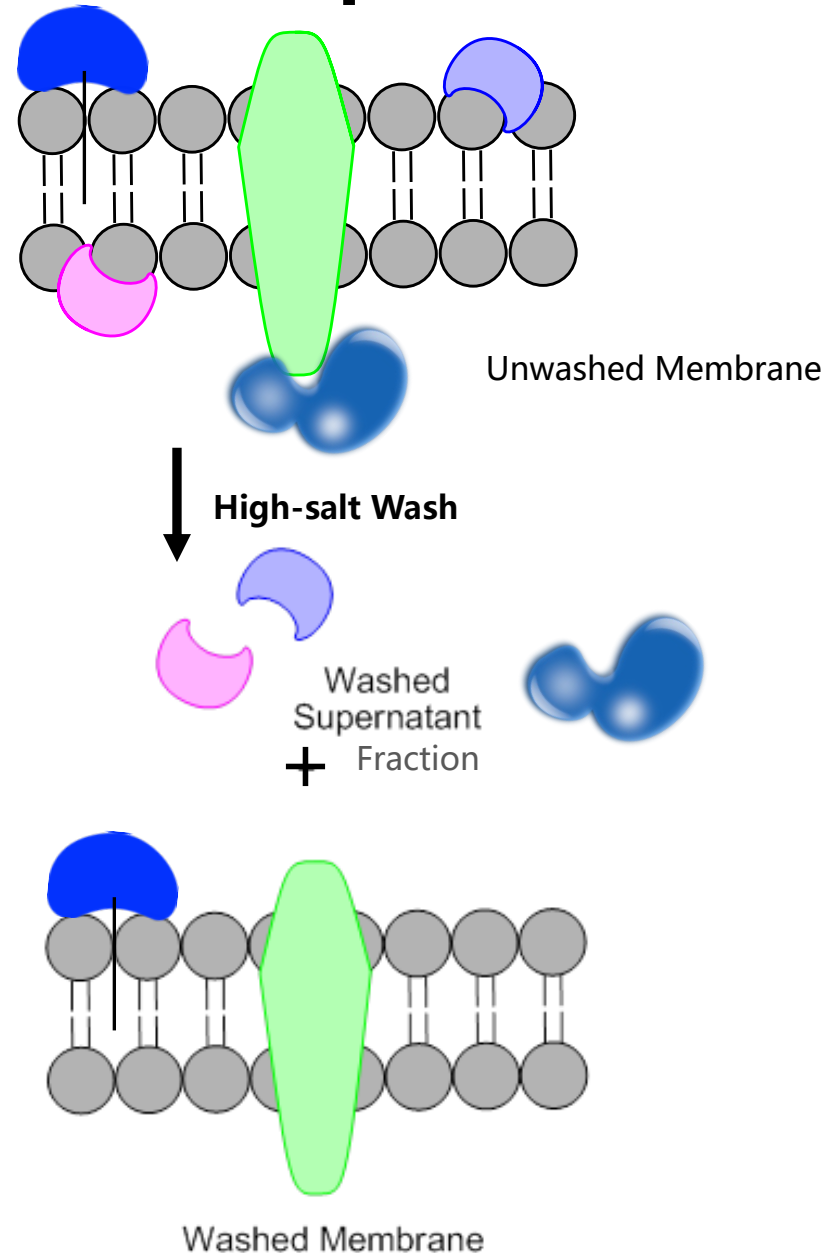


Isolation of Membrane Components

- Membrane components can be dissolved and separated by various solvents:
 - **Lipids** – organic solvent mixtures
 - **Carbohydrates** – typically found associated with proteins
 - **Proteins**
 - *Integral* – treating with detergents
 - *Peripheral* – washing with a high-salt buffer

Isolation of Membrane Components

- 3 Fractions:
- Unwashed Membrane
 - Peripheral and Integral
- Washed Membrane
 - Integral
- Washed Supernatant Fraction
 - Peripheral



Isolation of Membrane Components

- 3 Fractions:

- Unwashed Membrane

- Peripheral and Integral

- Washed Membrane

- Integral

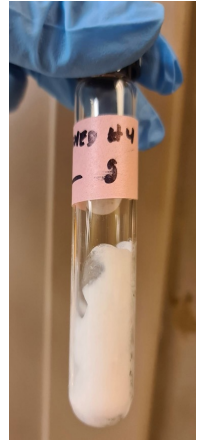
- Washed Supernatant Fraction

- Peripheral

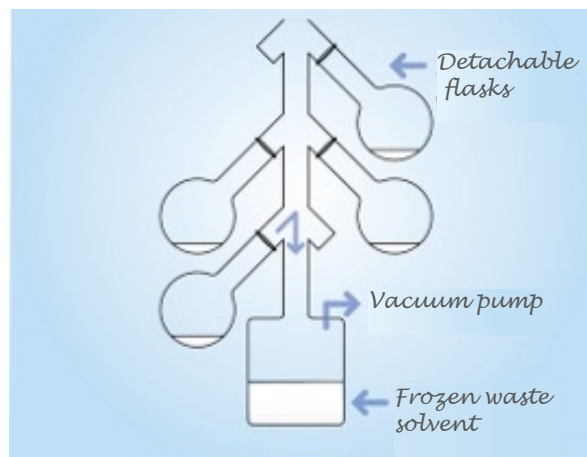
– Lyophilization

- Freeze drying of membrane preparations
- Removes water by sublimation
- Leaves all solids

– Solids will be used to extract lipids with organic solvents



LYOPHILIZATION



Freeze dried raspberries



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Part I: Isolating erythrocytes

Keep everything on ice!

Obtain bovine blood

60 mL blood + 20 mL PBS
Split into 2 conical tubes

Centrifuge for 10 min
(1000 x g)

Discard supernatant fraction (serum)

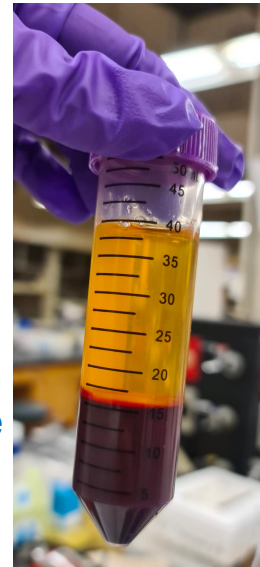
Keep pellet (= RBCs)

Resuspend in 20 mL PBS for each tube

Centrifuge for 10 min
(1000 x g)

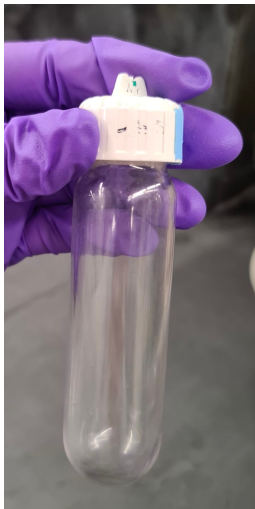
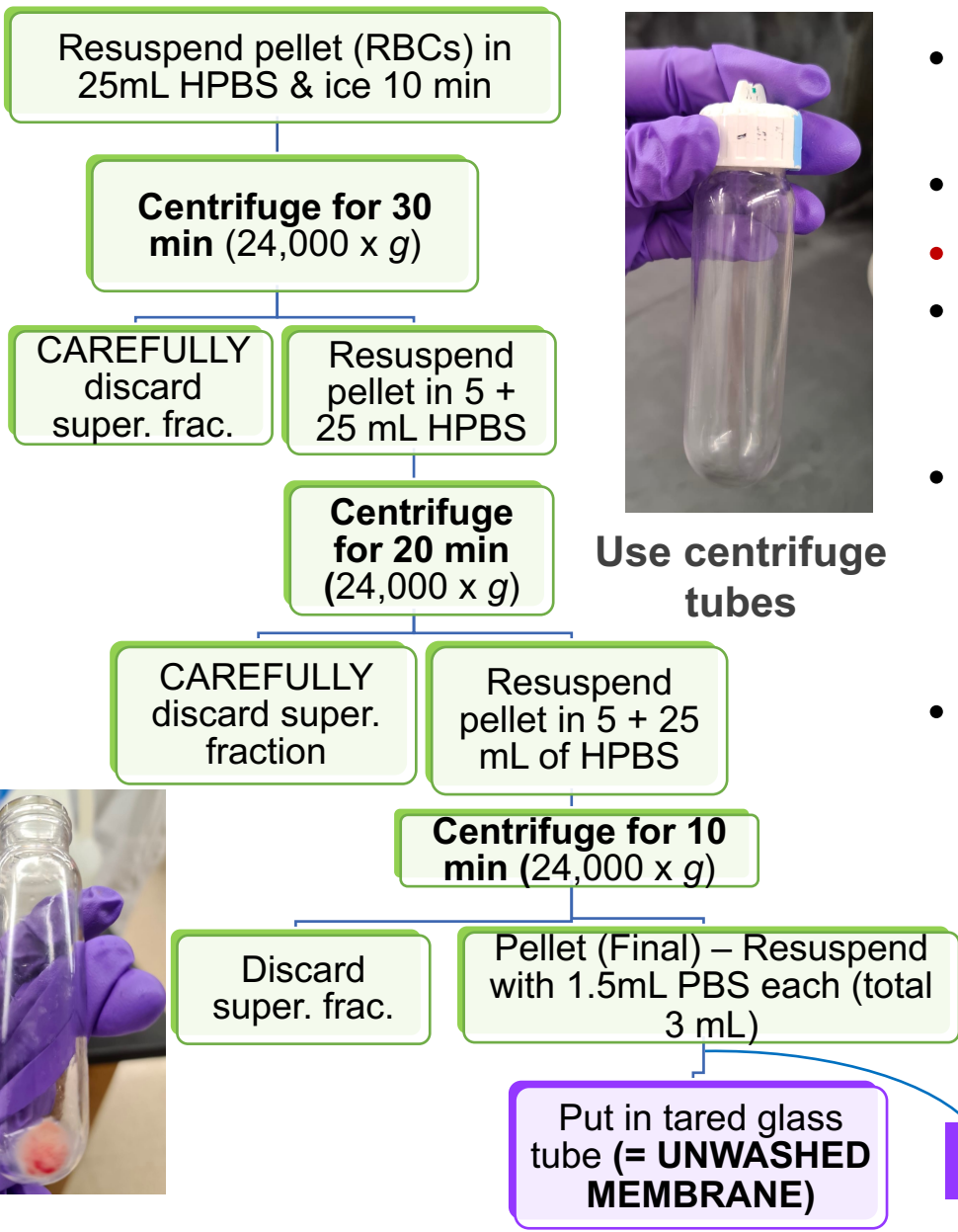
Discard supernatant fraction

Keep pellet (RBCs) for next step

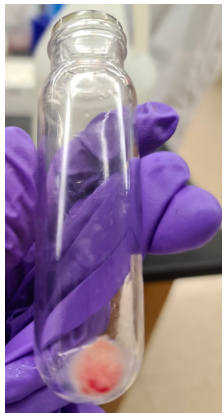


- Use clinical centrifuges with 50 ml conical tubes
- **Make sure to balance tubes!**
- **Make sure to use ONLY PBS – NOT HPBS!!!**
(Remember why??)
- **Some steps may be done in advance by TFs**

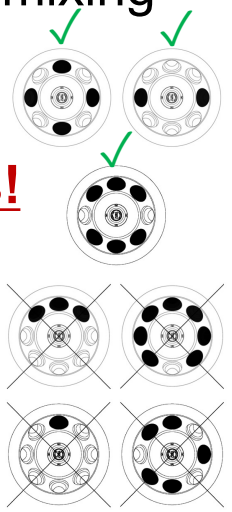
Part II: Isolating the plasma membrane



Use centrifuge tubes



- HPBS addition with gentle mixing to lyse cells
- Do not shake vigorously!
- **Balance centrifuge tubes!**
- Resuspend pellet with 5 ml HPBS & vortex, then add 25 ml more
- When resuspending membrane pellets, start with 0.75 mL of solution using a cut P1000 pipet tip
- Make sure you have empty weight of glass tube + label + cap on *analytical balance!*
 - Will need to measure change in mass of tube next week
 - Final fraction is **UNWASHED MEMBRANE**



Pull 25% aliquot

Part III: Washing the membrane

Use 1.5mL
microcentrifuge
tube

Remove 1 mL of
**UNWASHED
MEMBRANE**

Add 0.5 mL
MWB, mix, &
ice 10 min

**Centrifuge for 20
min (20,000 x g)**

Supernatant Fraction:
measure volume –
**WASHED
SUPERNATANT**

Pellet: Resuspend
in 1 mL PBS –
**WASHED
MEMBRANE**

Put in tared glass
tube (= **WASHED
MEMBRANE**)

Pull 25% aliquot

Pull 25% aliquot

- Save 25% aliquots of fractions & record volumes
 - **Unwashed**
 - **Washed**
 - **Supernatant Fraction**
- Freeze rest of fractions on dry ice – TFs will lyophilize before next week

CH 10A waste disposal

- Blood waste can go into a **separated** nearby collection carboy in the fumehood
- All other reagents are safe to dispose into the main steam waste carboy

Chapter 10a Lab Tips

Work efficiently between centrifugation steps! (there are 1 h 40 min of spin time)

Reminders for accurate taring

1. Pre-tape and write **ALL** information on tubes *and* caps **before** taring
2. Stick to one analytical balance for the remainder of Ch 10
3. Do not shake or move benches when weighing out tubes
4. Wait 5-10 seconds after shutting glass screen to record mass

At the end of lab you will turn in the following:

In tared labeled glass tubes (+caps) --

1. 75% unwashed membrane
2. 75% washed membrane

In 1.5 mL eppendorf tubes --

3. 25% aliquot of unwashed membrane
4. 25% aliquot of washed membrane
5. 75% aliquot of washed supernatant fraction
6. 25% aliquot of washed supernatant fraction

Don't forget to ALWAYS record your measurements of mass and volume when directed to do so in procedures

Chapter 10A clarification

- First step involves preparing 2 tubes of 30 mL bovine blood + 10 mL PBS
- Subsequent volume mentioned in the manual refers to each of the tubes
- Combine the two tubes of 1.5 mL each of the final pellet → UNWASHED membrane fraction
- Take note of the **change in centrifugation speeds and duration** that differs from the manual
- For the step involving removal of 1.0 mL of unwashed membrane preparation to 1.5 mL microcentrifuge tube, use the P1000 pipets. It should fit
- Remember to balance centrifuge tubes for ALL centrifugation steps

Chapter 10A

Before the lab period, you should have:

- ✓ Completed your Pre-lab Write-up and submit on Gradescope
 - ✓ Title, purpose and procedures
 - ✓ Remember to include:
 - ✓ Flowchart for your membrane isolation procedure
 - ✓ Note masses to record
 - ✓ Masses for tared Pyrex tubes for 75% washed & 75% unwashed membrane
 - ✓ Note volumes for all six fractions
 - ✓ (25% & 75%) x (Unwashed membrane, washed membrane, and washed supernatant fraction)

At the end of lab, you should have:

- ✓ Turned in your six LABELED!!!! aliquots to your TFs
 - ✓ Unwashed and washed membrane glass tubes, parafilm
 - ✓ Four eppendorf tubes and glass tube caps in a conical tube
- ✓ Washed your high-speed centrifuge tubes
(do not throw them away in the trash)



Clean →

Questions?

Survey & Discussion Quiz

